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PRINCIPAL INVESTIGATOR: Ralf-Peter Czekay, Ph.D.
Marilyn Farquhar, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Diego
La Jolla, California 92093

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Ralf-Peter Czekay, Ph.D.
Marilyn Farquhar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Diego
La Jolla, California 92093

e-mail:
rczekay@ucsd.edu

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13. ABSTRACT *(Maximum 200 Words)*

This proposal focuses on the question whether a derangement in expression or trafficking of scavenger receptors, LRP and megalin, might alter their interaction with uPAR, uPA, and PAI-1 in breast cancer cells. We established cell systems and prepared several essential reagents (antibodies) to evaluate the levels of scavenger receptor expression in three estrogen-insensitive and one sensitive breast cell lines, one normal mammary cell line, and a human fibrosarcoma cell line. Our novel findings demonstrate: 1) Differences in the level of expression and distribution of scavenger receptors, uPAR, uPA, and PAI-1 in normal vs. tumor cells. The increased expression of uPAR and uPA in cancer cells mimics the situation in vivo in breast carcinomas where overexpression of uPAR and uPA is associated with poor prognosis; 2) Surface uPAR and LRP colocalize into a molecular complex and are co-internalized via clathrin-mediated endocytosis; 3) Direct interaction between the two receptors stabilizes these complexes even after dissociation of uPA:PAI-1 in early endosomes; 4) LRP-dependent clearance of uPA:PAI-1 from the cell surface also requires uPAR binding to LRP. These results document functional interactions between scavenger receptors (LRP) and uPAR suggesting a role for LRP in the regulation of plasminogen activation in cancer cells.

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R.B. Ackley 10/12/99

PI - Signature Date

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PROGRESS REPORT

A. STATEMENT OF COLLABORATION

Work funded by this fellowship was done in the laboratory of Dr. Marilyn G. Farquhar. At the beginning of my funding period Dr. Farquhar also received funding through the U.S. Army Breast Cancer Research Program in the form of a Research grant (#DAMD17-96-1-6317). Since my fellowship only pays for my salary support, the expenses of my research were covered by Dr. Farquhar's grant. Thus there is a partial overlap between my Specific Aim (SA) #1 and the SAs #1 and #2 in Dr. Farquhar's proposal in regard to the subcellular localization of scavenger receptors, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), plasminogen activator inhibitor (PAI-1), and the receptor-associated protein (RAP). Dr. Kuemmel, a postdoctoral fellow working in Dr. Farquhar's laboratory, carried out the immunocytochemical studies, and I concentrated on the immunochemical aspects of our proposals during this second year of funding. Ms. Popa, a graduate student in our laboratory was involved in the preparation and initial characterization of anti-human megalin antibodies and in the purification of human alpha-2 macroglobulin. During the second year of our funding, she also participated in the cloning of human uPA and human PAI-1. Due to my experience and knowledge of the established immunochemical and immunocytochemical techniques in Dr. Farquhar's laboratory I was substantially involved in guiding Dr. Kuemmel and Ms. Popa through these protocols and in designing experiments necessary for their work on breast cancer cells. In addition, I introduced Dr. Kuemmel to Confocal Laser Scanning Microscopy and was actively involved in his training in this technique.

This decision to combine our effort increased our productivity and, as this report and Dr. Farquhar's report will clearly document, generated a significant amount of novel insight on the role of scavenger receptors in breast cancer cells and in particular about their role in the regulation of the uPA-system.

B. BODY OF WORK

I. INTRODUCTION

Proteases are of special importance in the pathogenesis of breast cancer because they play a key role in invasion and metastasis which requires the action of tumor-associated proteases to disrupt the tumor matix. Specifically, tissue concentrations of the urokinase-type plasminogen activator, uPA, its plasma membrane receptor, uPAR, and plasminogen-activator-inhibitor (PAI)-1 have been shown to have prognostic value in relationship to the progression of breast cancer (6, 31). Increased activity of uPA has been linked to cell migration and invasion during embryogenic development as well as to invasive growth and metastasis (6). Overexpression of uPA, its receptor, uPAR, and the uPA inhibitor, PAI-1, in primary tumors could be linked to an increased metastatic potential of these tumor cells.

Recent studies have reported that significantly higher levels of uPA and PAI:1 are found in mammary carcinomas than in their normal mammary epithelial cell counterparts (2, 8, 10, 12, 29, 32, 33) indicating a disturbance of the very delicate balance between activation and inactivation of uPA in tumor cells. It is known that this increase in tumor-associated proteases promotes invasion and metastasis through the dissolution of the surrounding basement membranes and tumor matrix.

The overall purpose of this work is to define the mechanisms responsible for the increased expression of PAI-1, uPA, and its receptor in breast cancers associated with increased potential for recurrence and metastasis. The working hypothesis to be tested is that the delicate balance between protease activation and inactivation/clearance is upset in breast

cancers with high metastatic potential. Recently discovered scavenger receptors, i.e., LRP and megalin (7, 16, 30), have been described to bind and to endocytose uPA:PAI-1 complexes (3, 14, 22, 24, 27, 35).

Both of these receptors are members of the LDL-receptor gene family, have a very similar overall structure in their extracellular domain and bind similar ligands in *in vitro* assays on cultured cells. Based on their ability to bind and clear uPA:PAI-1 complexes, these scavenger receptors are thought to play a significant role in biologic and pathologic processes involving tissue remodeling, i.e., embryonic development, wound healing, and malignant cell invasion.

The successful completion of the experiments proposed in the original application will shed light on a number of important questions including: 1) Is their a quantitative difference in the levels of scavenger receptor expression in normal mammary epithelial cells vs. breast cancer cells; 2) What are the trafficking itineraries of scavenger receptors and uPAR in both normal and tumor-derived cells; and 3) Are scavenger receptors involved in the down-regulation of cell surface uPA activities through co-internalization of uPA and uPAR? As a result of these studies we will gain insights as to the mechanisms responsible for the abnormal accumulation of uPA, its receptor and PAI-1 in breast tumors.

II. DETAILS OF PROGRESS

Our working hypothesis was that the increased expression of uPA, uPAR, and PAI-1 in breast cancers with high metastatic potential is due to abnormalities in the clearance of these molecules by scavenger receptors.

Work has been carried out under each specific aim as follows.

1. SPECIFIC AIM #1: Determine the Localization of LRP and Megalin in Normal and Tumor-derived Mammary Epithelial Cell Lines.

Background information from previous work:

Previous work has established that normal human mammary epithelial cells express either megalin or LRP (15, 36). At steady state the majority of the receptors are localized at the cell surface in clathrin-coated pits, whereas RAP is predominantly found intracellularly and in the rough ER (7, 18, 21, 26, 36). Except for our preliminary studies, the expression of scavenger receptors had not been studied in normal or tumor-derived mammary cell lines.

The majority of our objectives in this specific aim were accomplished during Years 1 and 2. We reached the following conclusions:

1) Immunochemical and immunocytochemical results demonstrated that normal and tumor cells express both LRP and RAP. Comparable amounts of RAP are produced by all normal and tumor cell lines, but LRP expression varies considerably.

2) Megalin, as determined by immunofluorescence (IF), was found to be expressed in all tumor cells (Hs578T>MDA-MB 231>MCF-7) but was not detected in a normal mammary cell line (184-B5).

3) The expression levels of LRP when compared between the tumor cell lines directly correlated with tumorigenicity in nude mice with MDA-MB 231 > Hs578T > MCF-7 cells.

4) The normal mammary cell line, 184-B5, expresses megalin, as does the tumor-derived cell lines MDA-MB 468, MDA-MB 231, and MCF-7. These results are in agreement with immunofluorescent studies accomplished in Year 1.

5) The tumor-derived cell line, MDA-MB 468, does not express LRP making it a useful cell line to study the functional relationship between megalin and components of the plasminogen system in the absence of LRP.

Additional Results Obtained in Year 3:

1. Localization of uPAR and LRP in HT1080 cells by immunofluorescence labeling (Confocal Laser Scanning Microscopy) and immunogold labeling:

Methods: Cells were prepared for immunocytochemistry as described under Specific Aim #2 in Dr. Farquhar's Progress Report #DAMD17-96-1-6317. For more details see legends to Figures #1, #2 and #3 in same Progress Report.

New Results for Specific Aim #3: To follow up our studies reported last year on the localization of LRP and uPAR in MDA-MB 231 and Hs578T breast cancer cell lines, we have investigated their distribution in a well-characterized metastatic cell type, HT1080, to determine if the results obtained for the breast cancer cell lines are shared with other malignant cell types. By immunofluorescence, we show that labeling for LRP significantly overlaps with a known marker for clathrin-coated pits (AP-2) in HT1080 cells, whereas uPAR demonstrates only partial colocalization with this marker. We were unable to find uPAR in caveolae by immunofluorescence or immunogold labeling in either Hs578T breast cancer cells or the human fibrosarcoma-derived cell line, HT1080. The results we have obtained by immunocytochemistry on Hs578T and HT1080 cells are similar to what we reported last year for MDA-MB 231 cells. These consistent and reproducible observations may reflect a common distribution pattern among various tumor-derived cell types. The overlapping distribution seen for LRP and uPAR, taken together with LRP largely being restricted to clathrin-coated vesicles, supports the idea that clearance of uPAR is LRP-dependent and further suggests that uPAR internalization requires a clathrin-coated microdomain.

For more details see Figures #1, #2 and #3 in Dr. Farquhar's Progress Report #DAMD17-96-1-6317.

Summary and Questions Answered: The results of these studies have clearly established: 1) which cell lines express LRP, megalin, and RAP, and where in the cells these proteins are located, 2) that there are differences between a normal mammary epithelial cell line and estrogen-sensitive and estrogen-insensitive tumor-derived cells in the levels of scavenger receptor expression, but not of RAP.

Our overall goal under Specific Aim #1 was to determine whether there are derangements between normal and tumor-derived cells in the expression or location of scavenger receptors that might be responsible for the higher levels of uPA, uPAR, and PAI-1 found in breast cancers. Our findings to date suggest that abnormal accumulation of uPA, uPAR, and PAI-1 cannot be explained by the absence of scavenger receptors since all tumor-derived cells express either LRP or megalin. We must therefore consider other possibilities--e.g., decreased expression of scavenger receptors at the cell surface or their mislocalization to different domains of the plasma membrane which might result in decreased clearance of uPA, uPAR, and PAI-1 and their accumulation at the cell surface. Based on these results we can conclude that there are variations in the level of scavenger receptor expression between normal and tumor cells, but additional data are necessary to understand how these variations might affect uPA, uPAR, and PAI-1 clearance.

2. SPECIFIC AIM #2: Determine the Levels of Expression of LRP and Megalin in Normal and Tumor-derived Mammary Epithelial Cells.

The majority of our objectives in this specific aim were accomplished during Years 1 and 2.

Summary: Our findings clearly indicate that the expression of LRP significantly varied between the normal and tumor-derived cells: 1) Total LRP expression in breast cancer cells is higher in estrogen-insensitive MDA-MB 231, which is the most metastatic and invasive cell line tested in our experiments; 2) LRP expression is the lowest in estrogen-sensitive MCF-7, which is the least aggressive cell line tested here; 3) LRP could not be detected in MDA-MB 468 breast cancer cells; 4) Megalin expression was found in the normal cells as well as in Hs578T and MDA-MB 468 cells using immunocytochemistry and immunoprecipitation; 5) uPAR was expressed in MDA-MB 231, MDA-MB 468, and Hs578T cells with the highest amount detected in MDA-MB 231 cells; 6) MDA-MB 231, Hs578T, and MCF-7 cells express LRP at the cell surface with MDA-MB 231 showing the highest amount of LRP; 7) HT1080 cells also show surface expression of LRP and uPAR and both receptors could be co-immunoprecipitated using anti-LRP antibodies.

Conclusion: Taken together, data gathered under Specific Aim #2 show that all breast cancer and the normal cell line express scavenger receptors to various levels. With the exception of MDA-MB 468 cells, all our cell lines express LRP. Interestingly, MCF-7 cells (estrogen-sensitive) and MDA-MB 468 cells (estrogen-insensitive) express none or only a very small amount of LRP and both are described as the least aggressive (metastatic) tumor-derived cell lines tested here. Whether there is a significance to this correlation between low or no expression of LRP and the lack of high metastatic potential has to be determined.

The fact that LRP and uPAR co-immunoprecipitate even in the presence of mild detergent suggests that there is a strong interaction between both receptors at the cell surface of HT1080 cells. This interaction of uPAR and LRP has been further investigated during the third year and additional evidence confirming the presence of uPAR:LRP complexes at the cell surface of tumor-derived cells will be shown under Specific Aim #4 in this report.

In addition, MDA-MB 468 cells with none or low expression levels of LRP but significant expression of megalin might present an ideal cell line to investigate the interaction of the uPA-system with megalin in particular. Current data concentrate solely on the role of LRP in the interaction of scavenger receptors with the uPA-system.

3. SPECIFIC AIM #3: Compare the Expression and Cellular Distribution of PAI-1, uPA, and uPAR in Relation to Scavenger Receptors in Normal Mammary Epithelial Cells vs. Tumor-derived Cell Lines.

The goal of these studies is to determine where in or on cells uPA, uPAR, and PAI-1 are located and whether they colocalize in the same organelles, especially whether they are found in the same microdomains of the cell membrane. By determining the precise subcellular localization of uPA, PAI-1, and uPAR, these studies will help us to better define their functional relationship in breast cancer cells with regard to localized cell surface proteolytic activities.

Background from previous work:

Breast cancer with high metastatic potential show increased expression of PAI-1 (5, 13, 29) and uPA (5, 7, 13) and both could be detected with immunohistochemical methods. In addition, PAI-1 could be located in the extracellular matrix (29). Distribution of uPAR in normal

mammary epithelial cells seems to be limited to focal adhesions. However, in MCF-7 cells it has been detected at the leading edge of the migrating cells.

During Years 1 and 2 we obtained the following findings based on combined approaches using immunocytochemical, biochemical, and molecular biological procedures:

1) All tested human breast cancer cell lines (estrogen-sensitive and insensitive) and the normal mammary cell line express uPAR on the cell surface and in the ER as shown by immunocytochemistry and immunobiochemistry.

2) In estrogen-insensitive highly malignant tumor cell lines (MDA-MB 231 and Hs578T cells) but not in normal cells occupied uPAR (uPA:uPAR) was mainly associated with focal adhesions. Unoccupied receptor (uPAR) was found always in a rather random distribution over the entire plasma membrane.

3) The expression levels of uPAR are greater in all tumor cell lines tested as compared to the normal mammary epithelial cell line which directly correlates with the situation in breast cancers *in situ*.

4) PAI-1 was not detected in the normal cell line but was abundantly expressed by the aggressive tumor cells (MDA-MB-231 and Hs578T) where it is deposited in the extracellular matrix.

5) Using RT-PCR we found that all cell lines tested--the normal mammary epithelial cell line (184-B5) and three tumor-derived cell lines (MCF-7, MDA-MB-231, and MDA-MB-468) express mRNA encoding full-length uPAR. In addition, RT-PCR amplification using primers flanking the three domains (D1, D2, and D3) of uPAR demonstrated that the mRNA encoding this receptor contains the complete information for expression of all three structural domains. These data confirm our immunoblotting results and provide evidence suggesting that uPAR in these cells is structurally complete and does not exhibit abnormal truncations.

6) In MDA-MB-231 and Hs578T cells, uPAR and LRP were both located on the plasma membrane by immunofluorescence in a punctate distribution and their distribution showed partial overlap. Localization at the EM level confirmed the partial colocalization along the plasma membrane, providing initial evidence that the two receptors can be found within proximity for a direct interaction.

7) Double immunocytochemical labeling for uPAR and caveolin, a well characterized marker for specialized plasma membrane microdomains called caveolae, demonstrated little or no overlap in their distribution by either immunofluorescence or immunogold labeling. This is in contrast to previous work that suggests GPI-anchored proteins, such as uPAR (1), are concentrated in caveolae.

8) By co-immunoprecipitation analysis we showed that uPAR and LRP are associated in a detergent-resistant complex at the cell surface which supports the hypothesis that clearance of uPAR occurs via an LRP-dependent pathway and requires complex formation with the scavenger receptor.

Additional Results Obtained in Year 3:

1. LRP and uPAR expressed on the surface of the breast cancer cell line, Hs578T, and a human fibrosarcoma, HT1080, are functionally active:

Methods: α2Macroglobulin (α2M) and AE78 peptide were radioiodinated using the Iodo-Bead method according to the manufacturer's instructions (Pierce). Iodinated protein or peptide was separated from free iodine by gel filtration chromatography.

For ^{125}I-α2M binding and internalization studies, Hs578T or HT1080 cells were incubated at 4°C for 4 h with ^{125}I-α2M (2 nM) in the absence or presence of varying amounts of unlabeled α2M (0.06 to 40 nM). Nonbound proteins were removed by rinsing, and cell associated radioactivity was quantitated by gamma counting and normalized to total cellular protein. Binding affinities (K_D) for ^{125}I-α2M were determined as the concentration of unlabeled α2M which resulted in a 50% inhibition of binding. Specificity was determined as the difference between total binding (without competition) and non-specific binding (non-competable). The amount of bound ligand was calculated as cpm divided by the specific activity of ^{125}I-α2M.

For ^{125}I-α2M internalization studies, Hs578T or HT1080 cells were incubated at 37°C with ^{125}I-α2M (2 nM) for 8 h in the absence or presence of unlabeled α2M (40 nM). Incubation medium was removed after 0, 2, 4, and 8 h, microfuged for 2 min, and supernatants were processed for trichloroacetic acid (10% TCA) precipitation and gamma counting. Degradation was calculated as TCA-soluble cpm/specific activity of the radioiodinated ligand and was normalized to total cellular protein.

For ^{125}I-AE78 binding studies, Hs578T and HT1080 cells were acid-washed with 0.2 M glycine, pH 3, at 4°C for 3 min to release endogenous bound uPA and uPA:PAI-1 from uPAR, followed by neutralizing with several rinses of PBS. Cells were then incubated with ^{125}I-AE78 (100 nM), a synthetic peptide that binds specifically to the uPA-binding site on uPAR (28), at 4°C for 4 h, after which the medium was removed, and cells were processed for gamma counting. Binding of ^{125}I-AE78 was competed by a co-incubation with unlabeled AE78 (10 μM) to determine specific binding. Results were normalized for total cellular protein.

Results: In our previous report, we show that LRP and uPAR are expressed on the surface of Hs578T breast cancer-derived cells. We now examined if the receptors are functionally active for ligand binding, and in the case of LRP, active also for ligand internalization. These studies were performed in parallel with HT1080 cells which are known to express functionally active uPAR on their surface. Using the LRP specific ligand α2M, we found that LRP in both cell types bound ligand with an apparent Kd of 0.75 nM **(Fig. 1)**. This value is in agreement with previous studies on various cell types (20). Moreover, LRP mediated rapid and continuous internalization of α2M as demonstrated by the time-dependent increase of intracellular degradation **(Fig. 2)**. uPAR in both cell types specifically bound similar amounts of its peptide ligand, AE78 **(Fig. 3)**. Together, these data demonstrate that both LRP and uPAR are functionally active on the surface of Hs578T cells, and their activity is comparable with the well-characterized tumor-derive cell line, HT1080.

4. SPECIFIC AIM #4: Determine the Fate of uPA, uPAR, Scavenger Receptors, and uPA:PAI-1 Complexes at the Cell Surface of Normal and Tumor-derived Mammary Epithelial Cells

Background from previous work:

Recently, a model has been proposed where uPA secreted by migrating cells will be activated when bound to uPAR at the cell surface. Activity of uPA is regulated and inactivated by complexing with PAI-1. Furthermore, it was suggested that uPA:PAI-1:uPAR complexes are then cleared from the cell surface via scavenger receptors (9, 11, 19, 23, 34). However, it is not known if uPAR and LRP are located together at the cell surface or if they are co-internalized. In addition, in breast cancers with metastatic potential increased expression of uPAR, uPA, and PAI-1 suggesting an abnormality in the binding and/or clearance of the complexes via scavenger receptors. The studies in this specific aim are designed to establish if ligand-occupied uPAR is co-internalized with LRP and/or megalin, and whether there are abnormalities in uPA:PAI-1 clearance by scavenger receptors (i.e., internalization and endocytic trafficking).

Summary of Findings Obtained in Years 1 and 2:

1) Cell surface radioiodination followed by immunoprecipitation analysis with anti-LRP antibodies showed that the number of receptors expressed on the two most malignant, estrogen-insensitive cell lines (MDA-MB-231 and Hs578T) is much greater than that which is detected on the estrogen-sensitive cell line (MCF-7). The relative amounts of LRP expressed on the surface of each breast cancer cell line can be summarized as MDA-MB-231>>Hs578T>>>MCF-7.

2) The amount of functional LRP expressed on the surface of each cell type as determined by quantitating ^{125}I-α_2M (α_2-macroglobulin) binding closely correlates with the quantitative data we obtained for LRP expression by immunoblotting and immunoprecipitation with MDA-MB-231 cells expressing much greater amounts (X10) of LRP than other cell lines.

3) Recycling of LRP after binding ligand takes place with normal kinetics in all cells tested except MDA-MB-231 cells which express a large number of functional LRP on the surface as evidenced by ligand binding studies, but show a low rate of ligand degradation. MDA-MB-231 cells thus appear to be unable to recycle LRP.

4) By cell surface radioiodinations, we found that MDA-MB-231 cells are capable of internalizing LRP as indicated by the decrease in cell surface labeled receptor after 10 min at 37°C. However, the amount of receptor measured on the cell surface remained unchanged over time, indicating that the internalized LRP is not recycled. Thus we have obtained evidence by two different methods of a defect in recycling in MDA-MB-231 breast cancer cells. LRP recycling kinetics were found to be normal for the non-tumor-derived mammary epithelial cell line (184-B5).

5) Immunoblotting and immunoprecipitation studies on MDA-MB-231 cell extracts indicated that uPAR is apparently truncated on cell surface. Analysis of uPAR mRNA by RT-PCR amplification showed that these cells express mRNA encoding full length uPAR, indicating that the truncation is not due to a genetic defect. We additionally found that MDA-MB-231 cells were unable to bind the uPAR-specific peptide ligand, AE78, suggesting that the truncated form of uPAR lacks Domain 1 of the receptor which contains the functional binding site for uPA. The absence of Domain 1 was confirmed by immunocytochemistry as uPAR anti-Domain 1 antibodies were unable to stain MDA-MB-231 cells, whereas anti-Domain 2 antibodies were able to readily detect uPAR on the cell surface.

6) We found that fluorescein-uPA bound specifically to the surface of Hs578T cells and in some cases was found in areas that are characteristic of focal contacts. After internalization of fluorescein-uPA during an 18°C temperature block to inhibit intracellular trafficking to late endosomes and lysosomes, we found uPA accumulated in early endosomes where it colocalized with Texas Red-labeled transferrin, a marker for clathrin-mediate endocytosis.

7) Subcellular fractionation studies showed that cell surface uPAR co-sediments with transferrin following endocytosis at 18°C. In addition, endocytosis of uPAR was significantly inhibited when the clathrin-mediated internalization pathway was blocked. Together, these results indicate that occupied uPAR is taken up via clathrin-coated vesicles and delivered to early endosomes.

Results Obtained in Year 3:

1. Internalization of uPAR into early endosomes is blocked by anti-LRP antibodies and RAP:

Methods: Holotransferrin (Tf) was used as a marker to identify gradient fractions enriched for either plasma membrane or early endosomes. Binding and internalization of ^{125}I-Tf by HT1080 cells, and subcellular fractionations using Percoll density gradients were

done precisely as described in our last progress report. Briefly, HT1080 cells were incubated with ^{125}I-Tf transferrin at 4°C for 1 hr or sequentially at 4°C and then 18°C for 1 hr each. After the 18°C incubation the remaining cell surface bound ^{125}I-Tf was released by acid washing followed by neutralization. Postnuclear supernatants were prepared, organelles were fractionated on Percoll density gradients, and fractions were processed for direct gamma counting.

For biotinylated uPAR studies, HT1080 cells were acid-washed in glycine buffer, pH 3, at 4°C for 3 min to release endogenous bound uPA and uPA:PAI-1 from uPAR. Cell surface proteins were biotinylated with 400 μM Biotin XX (Molecular Probes) at 4°C for 35 min. After quenching the biotin reaction with 20 mM glycine for 15 min, cells were incubated with preformed uPA:PAI-1 complexes in the absence or presence of either anti-LRP IgG (200 μg/ml) or RAP-GST (50 μg/ml) at 4°C and then 18°C for 1 hr each. uPA:PAI-1 complexes were prepared as previously described (4). Postnuclear supernatants were prepared and fractionated on Percoll density gradients. Fractions enriched for plasma membrane (#4-6) or early endosomes (#8-10) were separately pooled, proteins were extracted with CHAPS, and processed for immunoprecipitation of biotinylated uPAR using anti-uPAR mAb. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, probed with streptavidin-conjugated horseradish peroxidase (HRP) for 15 min at room temperature. Bound streptavidin was visualized by chemiluminescence detection.

Results: In our previous report, we showed data indicating that uPAR internalization is inhibited when the clathrin-mediated pathway is blocked by hypotonic shock and potassium depletion (17). These results suggested that uPAR is internalized through clathrin-coated vesicles, which is precisely the path taken by LRP. We have now followed up these studies to examine if LRP plays a direct role in clathrin-mediated internalization of uPAR. Using subcellular fractionation to clearly distinguish plasma membrane from early endosome containing fractions, we found that in the absence of anti-LRP or RAP, uPAR was internalized into early endosomes **(Fig. 4C)** which is consistent with our previous observation. However, in the presence of the ligand inhibiting anti-LRP IgG **(Fig. 4E)** or RAP **(Fig. 4D)**, uPAR remained associated with plasma membrane containing fractions even after 1 hour at 18°C. Our previous results, together with our new findings clearly demonstrate that LRP directly mediates the internalization of uPAR through the clathrin-coated vesicle pathway.

2. LRP and uPAR form detergent stable complexes following the binding of uPA:PAI-1 complexes:

Methods: Hs578T and HT1080 cells were incubated in the absence or presence of preformed uPA:PAI-1 complexes at 4°C for 2 h. Cells were extracted with CHAPS detergent and solubilized proteins were immunoprecipitated with anti-LRP mAb 11H4 at 4°C for 1 h. 11H4 specifically recognizes the C-terminal 13 amino acids of the cytoplasmic tail of human LRP. Antibody bound proteins were solubilized in Laemmli sample buffer supplemented with 10% b-mercaptoethanol, separated by SDS-PAGE, and immunoblotted with anti-uPAR pAb followed by chemiluminescent detection.

Results: When Hs578T breast cancer cells or HT1080 cells were incubated in the absence of uPA:PAI-1 complexes, we found little or no uPAR co-precipitating with LRP **(Fig. 5, lanes 1 and 3)**. However, when either cell type was incubated with uPA:PAI-1, a significant amount of uPAR was found to co-precipitate with LRP **(Fig. 5, lanes 2 and 4)**. These data indicate that LRP and uPAR form detergent-resistant, immunoprecipitable complexes and formation of these complexes is facilitated by the binding of uPA:PAI-1 complexes.

3. LRP and uPAR form a direct interaction on the cell surface:

Methods: For expression and purification of recombinant soluble uPAR, uPAR cDNA (I.M.A.G.E. Consortium, accession no. T75241) sequence encoding amino acids 1-274

(lacking the GPI-anchor site) was amplified by PCR using: 5'-primer, CCGGAATTCCCTGCGGTGCATGCAGTGTAAGACC, 3'-primer, CCCAAGCTTACTGC GGTACTGGACATCCAGGTC, and cycling parameters of 2 min at 94°C (1X), 30 s at 94°C, 1 min at 55°C, 1 min at 72°C (30X), 7 min at 72°C (1X). PCR product was subcloned into pET28b (EcoR1/HindIII, Novagen). E. coli strain BL21-DE3 expressing soluble uPAR was grown and induced as described (25). Bacteria were pelleted by centrifugation (2,500 x g, 10 min, 4°C), resuspended in 20 mM Tris, pH 7.4, 150 mM NaCl (TBS) containing 100 mg/ml lysozyme, and incubated for 1 h at 4°C. Equal volume of TBS containing 3% N-lauroyl sarcosine was added and incubation was continued for 1 h at 4°C. Lysates were cleared by centrifugation (15,000 x g, 20 min, 4°C) and supernatants were incubated with Ni^{2+}-affinity resin for 16 h at 4°C. Resin was washed with TBS, 1.5% N-lauroyl sarcosine followed by TBS, 0.1% N-lauroyl sarcosine. Bound uPAR was eluted with TBS, 0.1% N-lauroyl-sarcosine, 500 mM imidazole, and purified uPAR was dialyzed against TBS, 0.1% N-lauroyl-sarcosine (3X, 2 L).

For chemical crosslinking, ^{125}I-labeled recombinant uPAR (25 nM) was bound to HT1080 cells in serum free DME medium (supplemented with 2% BSA, 20 mM Hepes, pH 7.4) for 2 h at 4°C in the absence or presence of unlabeled uPAR (2.5 μM) or polyclonal anti-LRP IgG (200 μg/ml). Nonbound proteins were removed by rinsing and bound proteins were cross-linked by incubating cells with the thiol cleavable cross-linker DTSSP (1 mM) for 20 min at 4°C. The reaction was quenched with TBS for 10 min at 4°C and cell lysates were prepared in 10 mM CHAPS (1 h, 4°C). Immunoprecipitations were carried out on the lysates with anti-LRP mAb (11H4) and precipitated proteins were processed for SDS-PAGE followed by autoradiography and densitometry analysis.

Results: Formation of stable quaternary complexes between LRP, uPAR, and uPA:PAI-1 may occur by either a bridge between the two receptors mediated by an intermediate adaptor protein or by a direct interaction between LRP and uPAR. To determine if uPAR is able to directly bind to LRP, we attempted to bind and crosslink soluble recombinant uPAR to LRP on the surface of HT1080 cells. Our results show that anti-LRP antibodies were able to immunoprecipitate crosslinked ^{125}I-uPAR indicating a direct interaction between the two receptors **(Fig. 6, column 2)**. In the absence of crosslinker **(column 1)** or in the presence of excess unlabeled uPAR **(column 3)** or anti-LRP ligand blocking antibody **(column 4)**, little ^{125}I-uPAR was found crosslinked to LRP indicating the specificity of the interaction.

4. Detergent-resistant complexes formed between LRP and uPAR are stable following their internalization into early endosomes:

Methods: HT1080 cell monolayers were acid-washed in glycine buffer (50 mM glycine-HCl, pH 3.0, 100 mM NaCl) at 4°C for 3 min to release endogenous bound uPA from surface uPAR followed by rinsing in ice-cold PBS. Surface proteins were biotinylated with 400 μM Biotin-XX at 4°C for 35 min, and the reaction was quenched with 20 mM glycine in PBS, pH 7.4, for 15 min. Cells were then incubated with uPA:PAI-1 at 18°C for 1 h. Postnuclear supernatants were prepared and fractionated by centrifugation on Percoll gradients. Gradient fractions #8-10 (previously determined as containing early endosomes) were pooled. mAb 11H4, specific for the C-terminus of human LRP, was prebound to protein G-agarose beads and subsequently incubated with pooled fractions for 2 h at room temperature. After washing, immunoisolates were incubated at 4°C for 1 h in buffer containing 10 mM CHAPS (to solubilize membrane proteins), beads were separated from supernatant by centrifugation, and Percoll in the supernatant was pelleted by centrifugation at 100,000 x g for 1 h. Proteins from the 100,000 x g supernatant and material remaining on 11H4 antibody-bound agarose beads were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with anti-uPAR, anti-uPA, anti-LRP, and anti-Tf receptor antibodies followed by HRP-conjugated anti-mouse or anti-rabbit IgG and detection by enhanced chemiluminescence. The latter was depleted of crossreactivity against mouse IgG. Biotinylated proteins were detected using HRP-coupled avidin (ABC-Biotinylation Kit).

Results: Our results have thusfar showed that uPAR internalization into cells is dependent on LRP and mediated by clathrin-coated vesicles. Moreover, we showed that LRP and uPAR form detergent-resistant complexes suggesting a direct interaction is required for co-internalization. Our next objective was to investigate if the complex formed between LRP and uPAR is stable within the low pH environment of early endosomes following their endocytosis. Using subcellular fractionation and immunoisolation of LRP-enriched early endosomes, we show that uPAR remains associated with LRP following detergent extraction of the immunoisolated membranes **(Fig. 7, lanes 5 and 6)**. In contrast, co-internalized uPA:PAI-1 dissociated from the LRP/uPAR complex in early endosomes as it was found in the detergent soluble fraction **(Fig. 7, lane 3)** and not associated with LRP **(Fig. 7, lane 7)**.

5. uPAR and LRP both recycle from early endosomes (EE) to the plasma membrane (PM) in HT1080 cells:

Methods: Acid-washed confluent HT1080 cells were surface radioiodinated with 0.25 mCi Na-^{125}I/ml PBS at 4°C using the lactoperoxidase method. uPA:PAI-1 (50 nM) were bound to surface iodinated cells at 4°C for 1 h followed by incubation at 37°C for 0, 10, 20, or 30 min to allow internalization of uPA:PAI-1:uPAR:LRP complexes and recycling of the receptors. Incubations were stopped at 4°C and postnuclear supernatants were prepared and processed for subcellular fractionation using density gradient centrifugation. Fractions #4-6 (PM) and #8-10 (EE) were pooled, and radioiodinated LRP and uPAR were immunoprecipitated from both pools. Precipitated proteins were separated by SDS-PAGE, and radioactivity was quantitated by densitometry.

Results: We found that only 29% of the total ^{125}I-labeled LRP remained at the cell surface **(Figure 8B, open circles)** after 10 min at 37°C, indicating that 71% was internalized with an accompanying increase in the EE pool **(Figure 8B, closed circles)**. After 20 min, the amount of ^{125}I-labeled LRP at the PM **(Figure 8B, open circles)** increase to ~55% and at 30 min reached 63%. In the case of uPAR, we found that after 10 min incubation at 37°C **(Figure 8D, open circles)**, 53% of the ^{125}I-labeled uPAR remained at the cell surface accompanied by an elevation in the amount of uPAR in EE **(Figure 8D, closed circles)**. After 20 and 30 min the amount of uPAR at the cell surface increased to 62% and 75%, respectively, indicating recycling of a fraction of these receptors to the PM.

6. Quantitation of cell surface uPA-binding activity for uPAR on HT1080 cells:

Methods: In brief, confluent HT1080 cells were acid-washed to remove endogenously surface-bound uPA and uPA:PAI-1. Cells were incubated at 4°C with proteolytic active uPA in the presence or absence of proteolytic inactive pro-uPA. Specifically surface-bound active uPA was quantitated by a colorimetric assay using the synthetic substrate Chromozym U (Boehringer Mannheim).

In further experiments, acid-washed cells were incubated at 37°C with uPA:PAI-1 complexes in the presence or absence of RAP to prevent complex binding to LRP. After internalization of specifically bound complexes, cells were acid-washed and incubated at 4°C with active uPA to colorimetrically quantitate the amount of uPA-binding sites (uPAR) remaining at the cell surface after incubation with uPA:PAI-1.

Since LRP is able to reduce the overall levels of uPAR at the cell surface through endocytosis, pre-incubation with RAP should shift the equilibrium of LRP through endocytosis to internal pools, and allow endogenous uPAR to accumulate at the cell surface by recycling or de novo synthesis

For more details see Dr. Farquhar's Progress Report #DAMD17-96-1-6317.

Results: In brief, 1) Specific binding of active uPA to the cell surface of HT1080 cells shows that cell surface uPAR is functionally active for uPA binding; 2) Colorimetric analysis demonstrate the proteolytic activity of the uPAR-bound uPA; 3) Incubation with uPA:PAI-1

resulted in a 68% reduction in cell surface uPAR-bound uPA activity as compared to cells incubated without complexes, which is consistent with our previous results showing that uPA:PAI-1 complexes promote LRP-dependent co-internalization of uPAR; 4) In the presence of RAP, we measured a greater amount of uPAR-bound uPA activity indicating that RAP inhibited the LRP-mediated co-internalization of uPAR; 5) Pretreatment of cells with RAP does in fact increase the amount of uPAR-bound uPA activity by approximately 35%. These data further indicate that LRP is able to influence extracellular uPA activity by regulating the amount of uPAR present on the cell surface.

For further details see Figures #11, #12, and #13 in Dr. Farquhar's Progress Report #DAMD17-96-1-6317.

Summary of New Findings: In carrying-out the goals of Specific Aim #4, we have identified a sequence of events that describe the endocytic trafficking itinerary of LRP and uPAR, and address the functional consequences of their interactions. The details that follow are included in the model shown in **Figure 9**. The binding of uPA:PAI-1 complexes to cells facilitates an interaction between LRP and uPAR which is a result of direct binding of uPAR with bound ligand to LRP. After clathrin-mediated internalization of the quaternary complex (LRP/uPAR/uPA:PAI-1) into early endosomes, uPA:PAI-1 dissociates, while LRP and uPAR remain associated demonstrating the stability of their interactions. Internalization of uPAR by LRP removes potential uPA binding sites from the cell surface and effectively reduces the overall levels of uPA proteolytic activity. Internalized uPAR and LRP recycle to the plasma membrane. Although the major portion of our results have come from studies using HT1080 cells, many of the experiments were done in parallel with our breast cancer-derived cell lines with the same outcome.

IV. OVERALL CONCLUSIONS

General Statement: In the first year of this fellowship significant progress was made in characterizing the expression levels and subcellular distribution of the scavenger receptors (LRP and megalin), uPAR, uPA, and PAI-1 in 3 breast cancer cell lines (MDA-MB 231, Hs578T, and MCF-7 cells) and 1 normal mammary epithelial cell line (184-B5). We also made progress on each of the tasks outlined in the Statement of Work for the first year and have moved ahead of the original schedule in some cases (e.g., quantitative analysis of scavenger receptors). The most important general conclusion we could make at that point was that we had documented a number of differences between the cell lines studied in either expression levels of these proteins or their localization supporting our original working hypothesis --i.e., that scavenger receptors may be abnormal in breast cancer cells and that they are the missing link that needs to be thoroughly investigated in order to fully understand the connection between the high concentrations of uPAR, uPA and PAI-1 in mammary tumors and malignancies in breast cancer.

In the second year of our funding we made significant progress on Specific Aims #2, #3 and #4. We have obtained evidence that LRP and uPAR are localized together at the cell surface in several cell types and can be coprecipitated. We have also obtained evidence that uPAR and uPA are taken up into the cell by endocytosis via clathrin-coated vesicles. We have documented several abnormalities in the MDA-MB 231 cells--ie., they have a defect in recycling of LRP and they express a truncated form of uPAR. Again we have made progress on each of the tasks outlined in the Statement of Work.

In the third year of our award, we have completed the major goals outlined in Specific Aims #3 and #4. During the course of our investigations on the cell surface activities and endocytic trafficking of uPA, PAI-1, uPAR, and LRP, we have identified several important and novel criteria that define their functional relationship. The two most significant observations we report include; 1) the demonstration that LRP and uPAR directly interact on the cell surface which

defines the mechanism as to how LRP mediates the co-internalization of uPAR, and 2) LRP is able to control extracellular uPAR-bound uPA proteolytic activity by regulating the surface expression of uPAR through endocytosis.

Highlights of our results and conclusions to date are as follows.

- The scavenger receptors, LRP and megalin, are expressed in all tumor cell lines investigated except MDA-MB 468 cells which do not express detectable LRP. In the case of LRP, these receptors function normally in ligand binding. Megalin is expressed in all 3 breast cancer cell lines as well as the normal cell line by immunoblotting. The discovery of a lack of LRP expression in MDA-MB 468 cells provides us with an opportunity to study the role of megalin in uPAR uptake devoid of interference with LRP.

- There is a direct correlation between the level of LRP expression in breast cancer cell lines and tumorigenicity in nude mice in the cell lines studied so far. Quantitative analysis of LRP expression levels in the most malignant cell line studied (MDA-MB 231) by immunoblotting, immunoprecipitation, and ligand binding demonstrates that they express 10X greater levels of LRP than non tumor-producing breast cancer cells (MCF-7) or a normal mammary cell line (184-B5). Ligand binding and uptake studies suggest that although ligand binding to LRP is normal in MDA-MB 231 cells, receptor recycling is impaired.

- RAP is expressed at similar levels in both normal and tumor cell lines and is found in its usual location in the ER. Thus the distribution and expression of RAP are similar to other cell types of varied origin studied previously. From these data it is safe to conclude that the differences in the expression of scavenger receptors cannot be explained by differences in the level of expression of their chaperone, RAP.

- The levels of uPAR expression at the cell surface are considerably higher in the two more aggressive (estrogen-insensitive) breast cancer cell lines as compared to the estrogen-sensitive and normal cell line. This matches the finding that expression of uPAR is higher in more malignant breast tumors with increased potential for metastasis and indicates that the cultured cell lines provide a valid *in vitro* model to study the role of uPAR in breast cancer. Moreover, our studies to date suggest that occupied uPAR may have a different distribution in the more malignant cell lines. Unoccupied uPAR are seen along the entire cell surface in all cell lines, but in MDA-MB 231 and Hs578T cells, occupied uPAR were also associated with focal adhesion sites on the basal cell surface. This suggests that uPAR could play a significant role in localizing uPA proteolytic activity near focal contacts and thereby promote cell detachment and migration. We need to verify this finding by colocalization of uPAR at the EM level with uPA and focal adhesion markers such as integrin receptors, vinculin, paxillin and focal adhesion kinase.

- The protease, uPA, is found at the cell surface in all four cell lines. However, in the two estrogen-insensitive cell lines but not in normal cells, uPA is also found at focal adhesion sites. These results suggest that increased uPA proteolytic activity may exist at sites of cell adhesion which would greatly enhance cell detachment and migration of breast cancer cells as compared to normal cells.

- PAI-1 is a secreted protein that is known to associate with extracellular matrix (ECM) proteins. Our immunocytochemical results to date show that 1) PAI-1 is expressed in both estrogen insensitive breast cancer cell lines and is deposited in the ECM, and 2) it is not found in the ECM of the estrogen sensitive or normal cells. These results suggest that the expression of PAI-1 may directly correlate with the invasive phenotype of the most

aggressive breast cancer cell lines. We will perform quantitative studies to determine if any PAI-1 is being made by 184-B5 cells and other normal mammary cell lines and further evaluate PAI-1 expression levels in the breast cancer cells.

- uPAR and LRP were found to be colocalized at the cell surface and could be coprecipitated indicating their assembly into a complex at the cell surface. Unlike many other GPI-linked proteins, uPAR was not found in caveolae.

- Internalized uPAR with bound uPA colocalizes with transferrin and co-sediments with transferrin-containing vesicles indicating that the complex is taken up by endocytosis via clathrin-coated pits in at least some cell lines.

- Abnormalities were found in ligand degradation following LRP-mediated endocytosis in one of the tumor-derived cell lines (MDA-MB 231), and we have clearly demonstrated that this is due to a defect in LRP recycling. Moreover, we have shown that these cells have a truncated form of uPAR on their surfaces which is missing the uPA-binding Domain 1. The absence of Domain 1 may have a significant impact on uPA cell surface activities and clearance in these cells.

- Internalization of uPAR by clathrin-coated vesicles is inhibited by antibodies to LRP and by the ligand-binding antagonist, RAP, providing additional evidence that its clearance from the cell surface is LRP-mediated.

- The binding of uPA:PAI-1 to cells promotes the formation of detergent-resistant complexes between LRP and uPAR. Moreover, LRP/uPAR complexes remain associated following endocytosis into the low pH environment of early endosomes. The stability of the interaction between LRP and uPAR most likely leads to efficient clearance of the complex from the cell surface and thus removal of uPA:PAI-1 from the extracellular environment.

- The stable complex formed between LRP and uPAR is a result of a direct interaction. Previous studies, including ours during year 2, indicated that the endocytic clearance of uPAR from the cell surface is LRP-dependent. However, whether this co-internalization occurs by a direct interaction between the receptors, or rather by a bridging of the two receptors by an adaptor-like protein remained unknown. Using chemical crosslinking we show that LRP and uPAR form a direct and specific interaction on the cell surface, and thereby define how a stable complex is formed between LRP and uPAR.

- LRP-mediated internalization of uPAR reduces the amount of functional uPA-binding sites found on the cell surface, and thus reduces the overall levels of extracellular uPA proteolytic activity. Cell surface uPAR is known to serve as a binding and activation site for secreted pro-uPA, and this activation is required for subsequent activation of the plasminogen proteolytic cascade. We show that inhibition of LRP-mediated clearance of uPAR increases the amounts of uPAR present on the cell surface. These data suggest that LRP is able to control extracellular uPA activity by regulating surface expression of uPAR through endocytosis.

- After internalization of LRP:uPAR complexes into EE both uPAR and LRP recycle to the cell surface. The recycling kinetics of uPAR are in agreement with previous findings that GPI-anchored proteins recycle more slowly to the PM than transmembrane receptors such as the LDL and transferrin receptors. We also found that the time course of LRP recycling in the tumor cell lines is slower than that reported previously for non-cancer cells. This indicates

that dissociation of the two receptors must occur in EE after release of bound uPA:PAI-1 or in sorting endosomes. However, after subsequent separation of LRP from uPAR, LRP recycles more rapidly to the PM than uPAR.

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APPENDICES

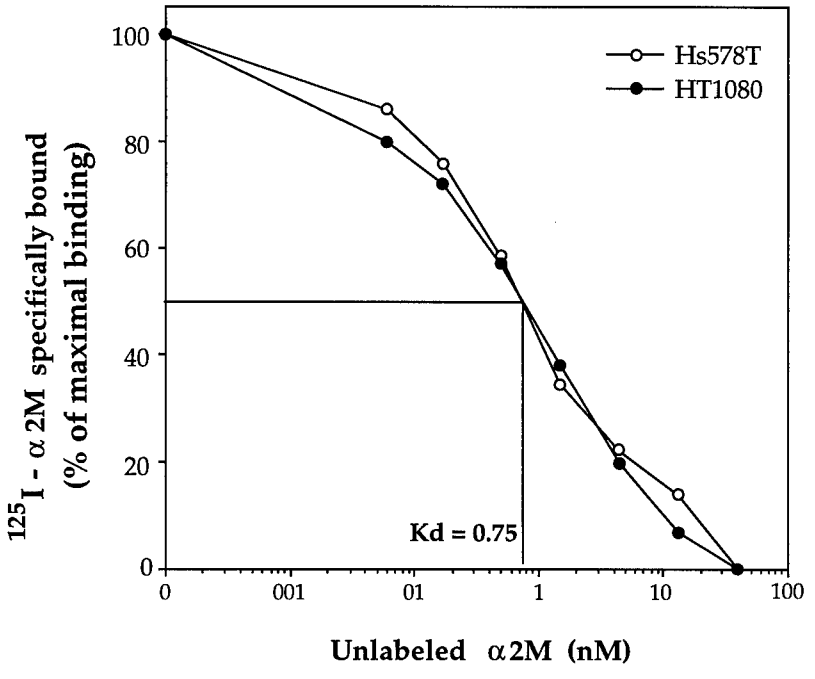

Fig. 1. LRP expressed by HT1080 and Hs578T cells is functionally active: Binding affinity for α2-macroglobulin. Cells were incubated with 125I-labeled α2M (2 nM), an LRP-specific ligand, at 4°C in the absence or presence of varying amounts of unlabeled α2M (0.06-40.0 nM). Cell associated radioactivity was quantitated by gamma counting, and ligand binding affinity was calculated as described in Methods. HT1080 cells (closed circles) and Hs578T cells (open circles) show the same affinities for α2M, KD=0.75 nM.

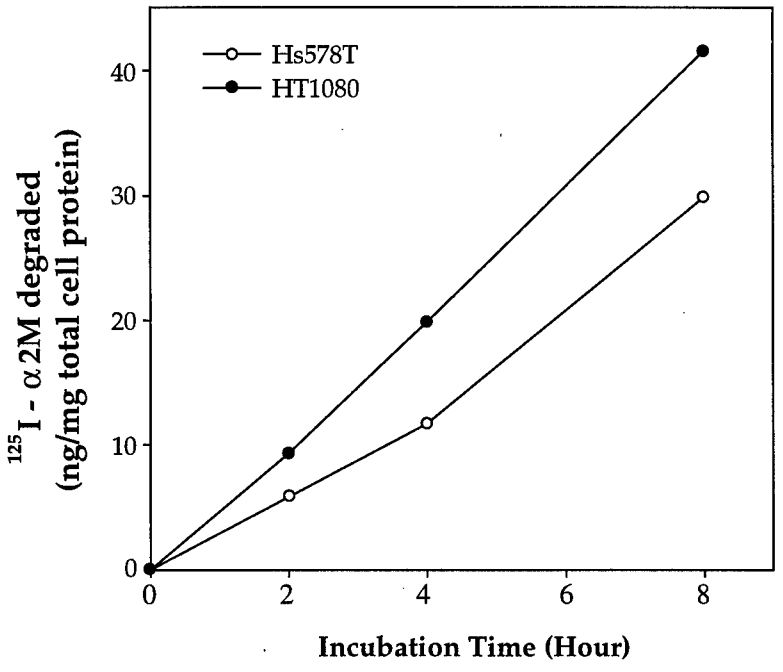

Fig. 2. LRP expressed by HT1080 and Hs578T cells is functionally active: Endocytosis of α2-macroglobulin. Cells were incubated at 37°C with 125I-labeled α2M (2 nM) for the indicated times, and the TCA-soluble radioactivity released into the incubation medium was assessed. After 8 h HT1080 (closed circles) and Hs578T (open circles) cells degraded 41.5 ng 125I-labeled α2M and 29.9 ng 125I-labeled α2M/mg total cell protein, respectively, indicating that both cell types express LRP and that these receptors take up and degrade ligand.

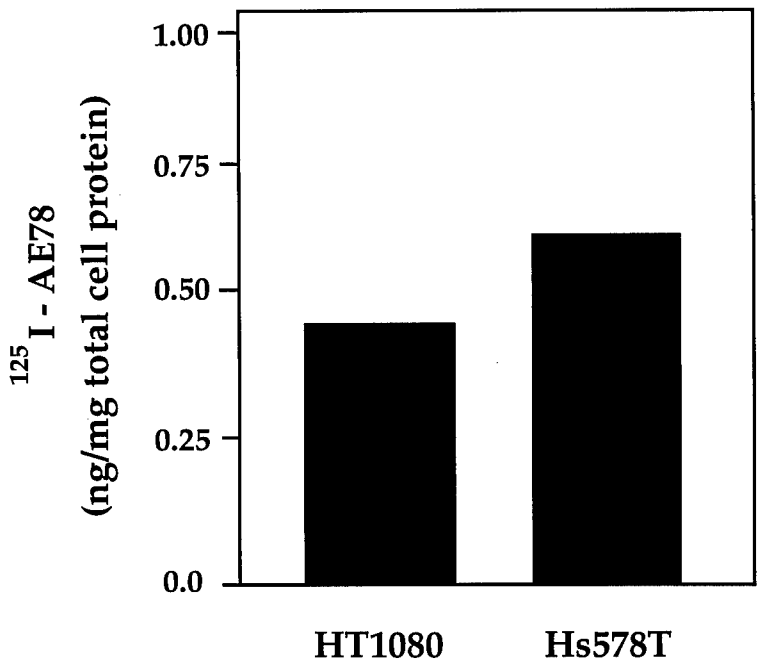

Fig. 3. AE78 binds to uPAR expressed on the surface of HT1080 and Hs578T cells. Cells were acid-washed and incubated with 125I-labeled AE78 (100 nM), in the absence or presence of 50x excess (5 μM) unlabeled AE78 for 4 h at 4°C. 125I-labeled AE78 specifically bound to the cell surface was determined. HT1080 and Hs578T cells bind similar amounts of 125I-labeled AE78, i.e., 0.41 ng and 0.68 ng/mg total cell protein, respectively, indicating that uPAR expressed by these cells is capable of binding uPA.

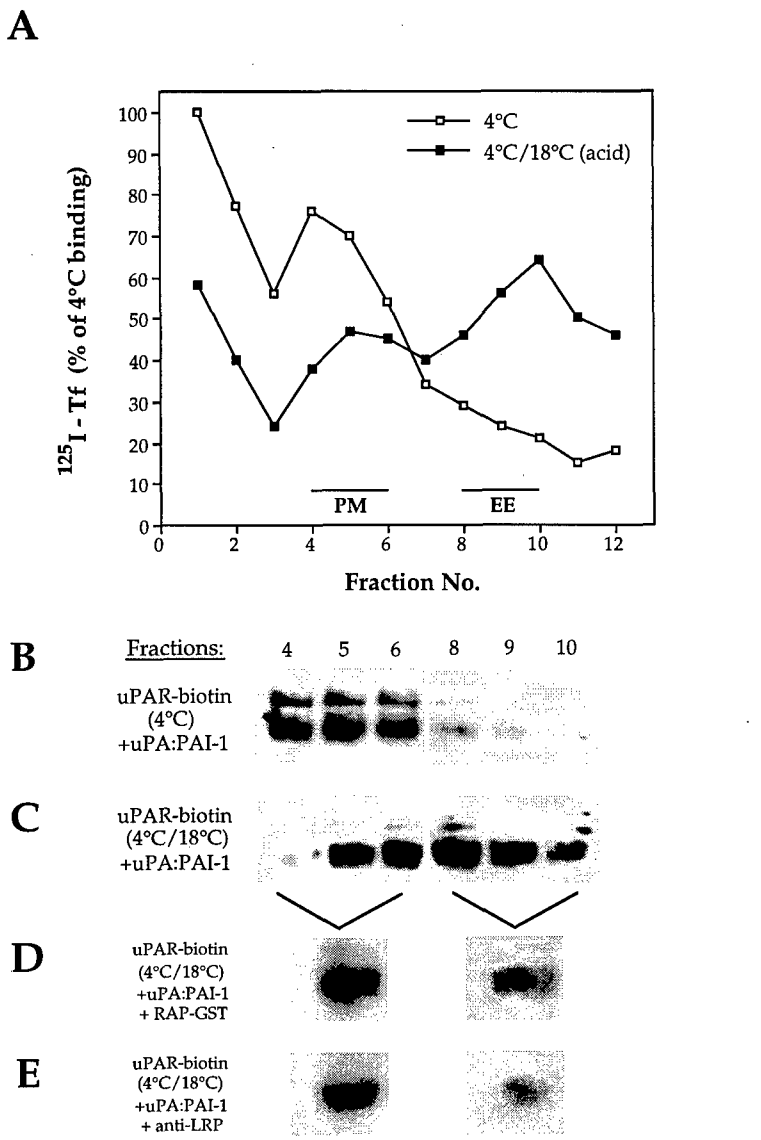

Fig. 4. Internalization of uPAR by HT1080 cells is LRP-dependent. Cells were surface biotinylated and incubated with either 125I-labeled Tf (200 ng/ml) or uPA:PAI-1 complexes (50 nM) at 4°C (60 min) or for 4°C (60 min) followed by 18°C (60 min). Postnuclear supernatants were fractionated on Percoll gradients, and fractions were processed for gamma counting of 125I-labeled Tf or for immunoprecipitation of uPAR followed by detection of biotinylated uPAR by blotting with HRP-coupled avidin. (A) In cells incubated at 4°C (open squares), 125I-labeled Tf peaks in fractions #4-6 indicating the location of plasma membrane (PM). After incubation at 18°C (closed squares), 125I-labeled Tf peaks in fractions #8-10 where it defines the location of early endosomes (EE). (B) In cells incubated at 4°C with uPA:PAI-1, biotinylated uPAR sediments in PM fractions #4-6. (C) After 60 min incubation at 18°C, the distribution of a substantial fraction of the biotinylated uPAR shifts to EE fractions (#8-10). After incubation for 60 min at 18°C in the presence of 30 μg/ml RAP-GST (D), or 200 μg/ml anti-LRP pAb (456) (E), the majority of the biotinylated uPAR is associated with PM fractions. PM fractions (#4-6) and EE fractions (#8-10), respectively, were pooled prior to analysis in "D" and "E".

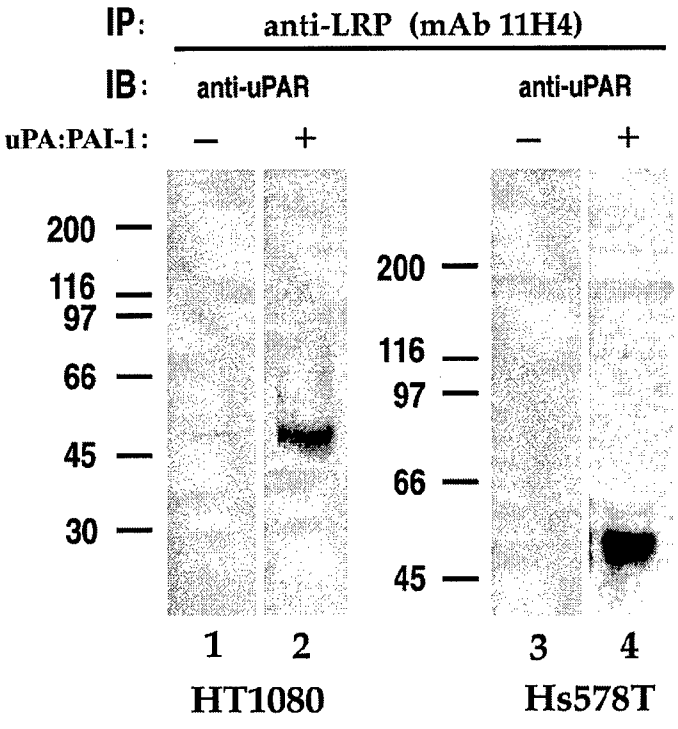

Fig. 5. LRP and uPAR form detergent stable complexes at the surface of HT1080 and Hs578T cells in the presence of uPA:PAI-1. Cells were incubated in the presence or absence of uPA:PAI-1 and processed for immunoprecipitation with anti-LRP mAb (11H4). Precipitated proteins were subsequently immunoblotted (IB) with polyclonal anti-uPAR (465). In the presence of uPA:PAI-1, immunoprecipitates from both cell lines contain uPAR (lanes 2 and 4) indicating co-immunoprecipitation of uPAR with LRP. In the absence of uPA:PAI-1 (lanes 1 and 3), only traces of uPAR could be detected in immunoprecipitates.

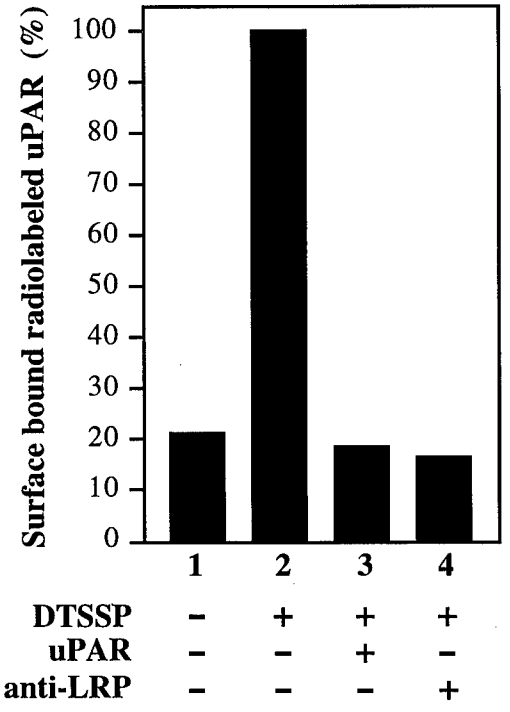

Fig. 6. Direct binding of uPAR to LRP at the cell surface of HT1080 cells. Acid-washed cells were incubated with soluble 125I-labeled uPAR (25 nM) at 4°C in the presence or absence of unlabeled soluble uPAR (2.5μM) or polyclonal anti-LRP IgG (456) (200 μg/ml). Bound 125I-labeled uPAR was cross-linked in the presence (columns 2-4) or absence (column 1) of DTSSP (1 mM) for 20 min at 4°C, proteins were solubilized with CHAPS, and immunoprecipitated with anti-LRP mAb (11H4). Antibody bound proteins were processed for autoradiography and densitometry. The total amount of 125I-labeled uPAR cross-linked in the absence of competitors was set to 100% (column 2). In the presence of unlabeled uPAR (column 3) or anti-LRP IgG (column 4), the amount of cross-linked 125I-labeled uPAR was significantly reduced (18.5% and 16.3%, respectively), indicating that uPAR can bind directly to LRP.

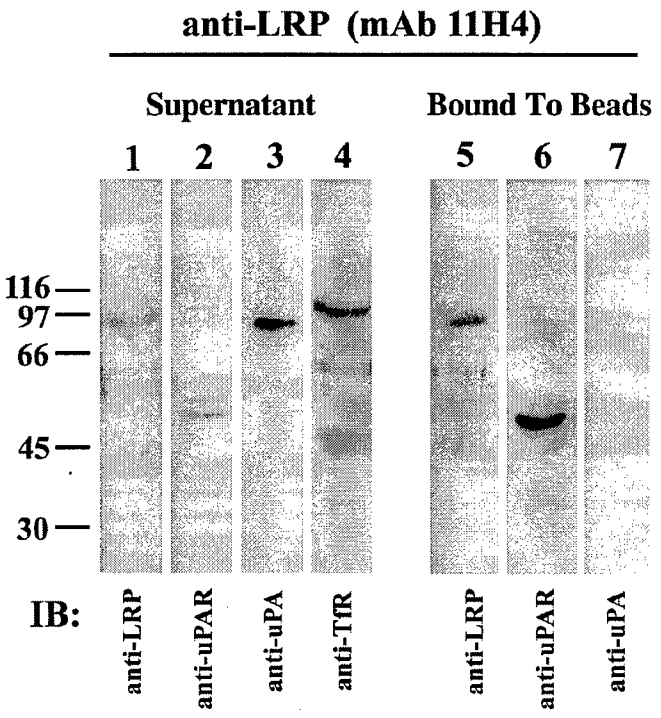

Fig. 7. LRP and uPAR complexes are present in EE. uPA:PAI-1 was bound to biotinylated HT1080 cells at 4°C, and the cells were fractionated as in Fig. 2. EE fractions were pooled and LRP-containing vesicles were immunoisolated using mAb 11H4 (against the cytoplasmic tail of LRP) that had been bound to protein G-agarose beads. Beads were treated with 10 mM CHAPS and proteins released into the supernatant and those associated with beads were analysed by immunoblotting with anti-LRP (1073), anti-uPAR (465), anti-uPA, and anti-Tf receptor. Only traces of both, LRP (lane 1) and uPAR (lane 2) could be detected in the CHAPS-supernatant, whereas the majority of uPA:PAI-1 complexes (lane 3) and Tf receptor (lane 4) was released into the supernatant. Immunoblotting of proteins released from the CHAPS-pellet showed that the majority of LRP (lane 5) as well as uPAR (lane 6) could be found in this pellet which contained only trace amounts of uPA:PAI-1 (lane 7). Thus uPAR and LRP form a CHAPS-resistant complex that can be co-immunoprecipitated from EE.

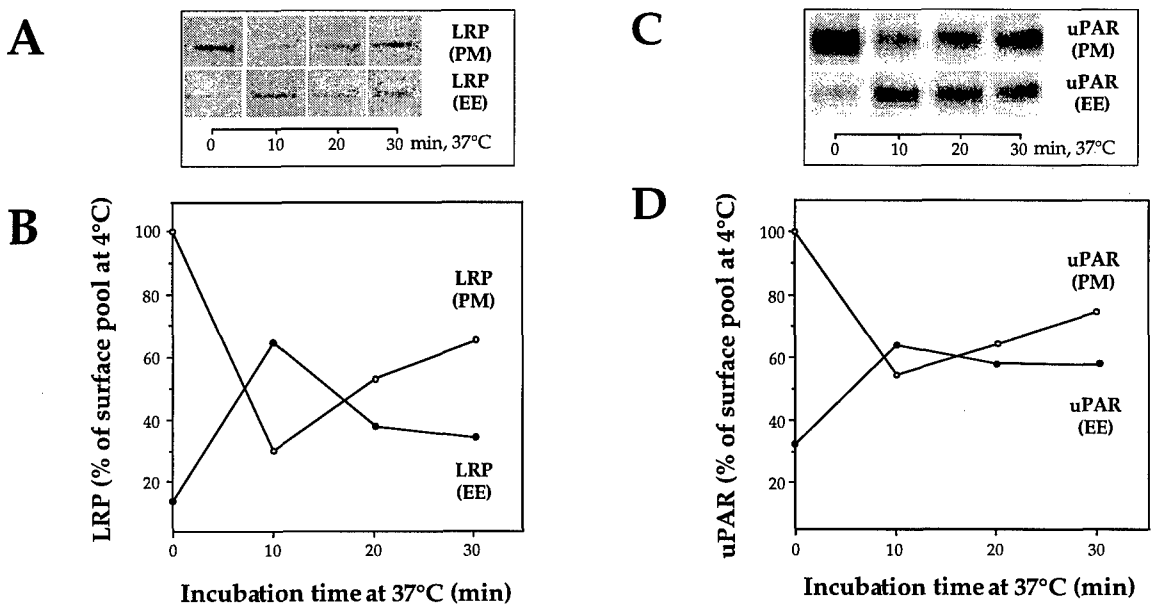

Fig. 8. LRP and uPAR recycle to the cell surface in HT1080 cells. Acid-washed cells were radioiodinated, incubated with uPA:PAI-1 at 37°C for 0, 10, 20, and 30 min, and processed for fractionation as in Figure 4. Immunoprecipitations were carried out on pooled PM and EE fractions with mAbs against LRP and uPAR. Proteinswere separated by SDS-PAGE, visualized by PhosphorImager analysis (A and C), and quantitated by densitometry (B and D). By definition, at 0 min, 100% of the 125I-labeled LRP (B, open circles) and 125I-labeled uPAR (D, open circles) were found at the PM. After 10 min at 37°C, only 29% of the LRP remains at the cell surface (B, open circles) indicating that 71% of the receptor has been internalized. After 20 and 30 min 55% and 63%, respectively, is found at the cell surface (B, open circles). The amounts of 125I-labeled LRP detected in EE (B, closed circles) are increased after 20 and 30 min. For uPAR, 53% of the receptor is found at the PM (D, open circles) after 10 min, indicating that 47% of the receptor has been internalized. At 20 min (62%) and 30 min (75%) increasing amounts of 125I-labeled uPAR are found at the PM (D, open circles). The amounts of 125I-labeled uPAR in EE (D, closed circles) at 10 min (62%), 20 min (56%), and 30 min (57%) increase over the time course of incubation at 37°C.

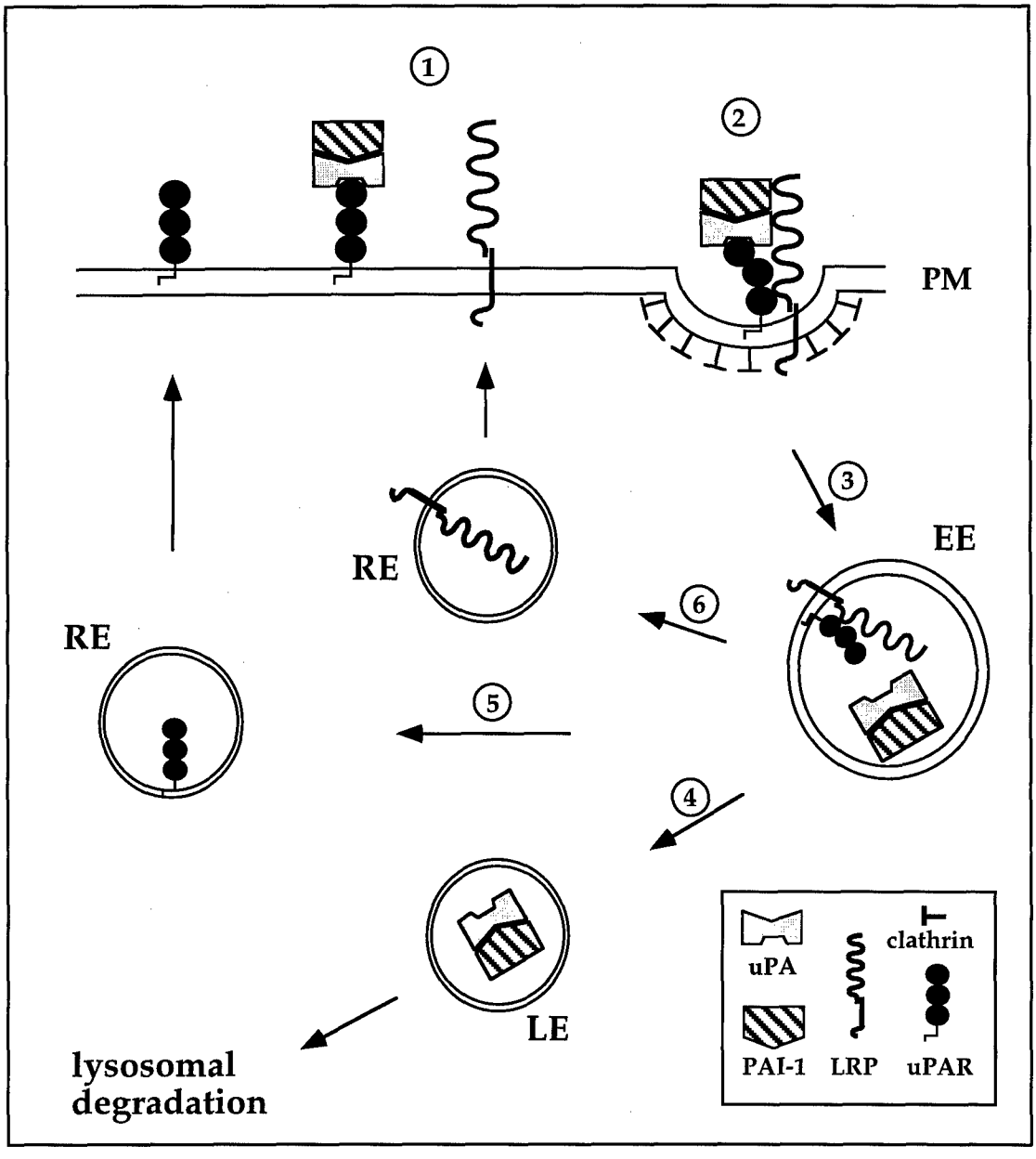

Fig. 9. Model depicting the clathrin-mediated LRP-dependent internalization of uPA:PAI-1 and uPAR. (1) uPAR-bound uPA:PAI-1 binds to LRP promoting a stable interaction between uPAR and LRP. Subsequently, these quaternary complexes are internalized via clathrin-coated vesicles (2) into early endosomes (EE) (3). After dissociation from uPAR in EE, uPA:PAI-1 traffics to late endosomes (LE) (4) and ultimately to lysosomes for degradation. uPAR (5) and LRP (6) also dissociate and traffic to recycling endosomes (RE) from which they return to the cell surface with different time courses.

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RALF-PETER CZEKAY, PH.D., ASSISTANT PROJECT SCIENTIST
DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE
MAIL CODE 0651

9500 GILMAN DRIVE
LA JOLLA, CALIFORNIA
92093-0651

Commander
U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, MD 21702-5012

October 12, 1999

Dear Sir,

I like to indicate with this letter that my Progress Report 1999 (DAMD17-96-1-6318) contains unpublished data regarding Specific Aims #1-#4. Therefore, I like to request that all presented results in this report may be limited for public release.

However, the data generated during Years 1-3 of this funding period were assembled into two manuscripts which have been submitted to peer-reviewed scientific journals. As soon as these manuscripts are accepted for publication I will submit copies for your files.

Sincerely,

Ralf-Peter Czekay, Ph.D.

Telephone: (858) 534-7713
FAX: (858) 534-8549
e-mail: rczekay@ucsd.edu

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BERKELEY • DAVIS • IRVINE • LOS ANGELES • RIVERSIDE • SAN DIEGO • SAN FRANCISCO SANTA BARBARA • SANTA CRUZ

RALF-PETER CZEKAY, PH.D., ASSISTANT RESEARCH SCIENTIST
DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE
MAILCODE 0651

9500 GILMAN DRIVE
LA JOLLA, CALIFORNIA
92093-0651

Commander
U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, MD 21702-5012

October 12, 1999

Dear Sir,

After three years of well funded breast cancer research this Progress Report is also my Final Report for my Breast Cancer Research Fellowship. I like to take this as an opportunity to thank the DOD Breast Cancer Research Program for this support which marked an important step in achieving my goal to establish myself as a competitive researcher in the field of cancer related research, especially breast cancer.

Your generous funding allowed me to present my research results at three national scientific meetings and to discuss my findings with an international audience. My efforts also resulted in the preparation and recent submission of two manuscripts to peer-reviewed scientific journals.

Sincerely,

Ralf-Peter Czekay, Ph.D.

Telephone: (858) 534-7713
FAX: (858) 534-8549
e-mail: rczekay@ucsd.edu

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 19 Jan 01

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-96-1-6318. Request the limited distribution statement for Accession Document Numbers ADB241037, ADB257719 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management